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# Recombinant 55-kDa Tumor Necrosis Factor (TNF) Receptor

STOICHIOMETRY OF BINDING TO TNF $\alpha$  AND TNF $\beta$  AND INHIBITION OF TNF ACTIVITY\*

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The extracellular domain of the 55-kDa TNF receptor (rsTNFR $\beta$ ) has been expressed as a secreted protein in baculovirus-infected insect cells and Chinese hamster ovary (CHO)/dhfr<sup>-</sup> cells. A chimeric fusion protein (rsTNFR $\beta$ -h $\gamma$ 3) constructed by inserting the extracellular part of the receptor in front of the hinge region of the human IgG C $\gamma$ 3 chain has been expressed in mouse myeloma cells. The recombinant receptor proteins were purified from transfected cell culture supernatants by TNF $\alpha$ - or protein G affinity chromatography and gel filtration. In a solid phase binding assay rsTNFR $\beta$  was found to bind TNF $\alpha$  with high affinity comparable with the membrane-bound full-length receptor. The affinity for TNF $\beta$  was slightly impaired. However, the bivalent rsTNFR $\beta$ -h $\gamma$ 3 fusion protein bound both ligands with a significantly higher affinity than monovalent rsTNFR $\beta$  reflecting most likely an increased avidity of the bivalent construct. A molecular mass of about 140 kDa for both rsTNFR $\beta$ -TNF $\alpha$  and rsTNFR $\beta$ -TNF $\beta$  complexes was determined in analytical ultracentrifugation studies strongly suggesting a stoichiometry of three rsTNFR $\beta$  molecules bound to one TNF $\alpha$  or TNF $\beta$  trimer. Sedimentation velocity and quasielastic light scattering measurements indicated an extended structure for rsTNFR $\beta$  and its TNF $\alpha$  and TNF $\beta$  complexes. Multiple receptor binding sites on TNF $\alpha$  trimers could also be demonstrated by a TNF $\alpha$ -induced agglutination of latex beads coated with the rsTNFR $\beta$ -h $\gamma$ 3 fusion protein. Both rsTNFR $\beta$  and rsTNFR $\beta$ -h $\gamma$ 3 were found to inhibit binding of TNF $\alpha$  and TNF $\beta$  to native 55- and 75-kDa TNF receptors and to prevent TNF $\alpha$  and TNF $\beta$  bioactivity in a cellular cytotoxicity assay. Concentrations of rsTNFR $\beta$ -h $\gamma$ 3 equimolar to TNF $\alpha$  were sufficient to neutralize TNF activity almost completely, whereas a 10-100-fold excess of rsTNFR $\beta$  was needed for similar inhibitory effects. In view of their potent TNF antagonizing activity, recombinant soluble TNF receptor fragments might be useful as therapeutic agents in TNF-mediated disorders.

Tumor necrosis factor (TNF)<sup>1</sup>  $\alpha$  and  $\beta$  are two closely related cytokines with about 30% sequence homology (1-3). Their genes are closely linked in the major histocompatibility complex of mammals (4). TNF $\alpha$  and TNF $\beta$  are primarily produced by activated macrophages and lymphocytes, respectively (5, 6). Based on crystallographic (7, 8) and analytical centrifugation studies (9) both cytokines are believed to form trimers. A wide variety of TNF $\alpha$  and TNF $\beta$  activities *in vitro* has been described including growth enhancement of fibroblasts (10), growth inhibition or lysis of some transformed cells (11), differentiation of human myeloid cell lines (12), and induction of the expression of cell surface molecules (13-15). *In vivo* TNF $\alpha$  induces hemorrhagic necrosis of certain transplantable tumors (16, 17), is involved in immune and inflammatory reactions (18-20), and mediates lethal effects in endotoxin-induced septic shock (21-23).

We have identified two human TNF receptors of about 75- and 55-kDa apparent molecular masses (in the present paper called TNFR $\alpha$  and TNFR $\beta$ , respectively) by chemical cross-linking with radiolabeled TNF $\alpha$  (24) and by binding of monoclonal antibodies generated against isolates of the receptors (25). Subsequently, both receptors have been purified from HL60 cells and partial amino acid sequences were determined (26). More recently, the cDNAs of TNFR $\alpha$  and TNFR $\beta$  were isolated by us (27, 28) and several other groups (29-34). The two receptors show similar cysteine-rich motifs in their extracellular domains and belong to a new cytokine receptor gene family which includes the nerve growth factor receptor, CD40, and OX40 antigens (28, 35).

Soluble fragments of both TNF receptors have been found to be present in human serum and urine (36-40). In certain disease states receptor shedding appears to be increased (40, 41). Soluble TNF receptors have also been identified in cell culture medium of some transformed cell lines (32, 42) and of stimulated polymorphonuclear leukocytes (43). In functional studies the natural TNF receptor fragments have been shown to protect cells from TNF $\alpha$ -induced cytotoxicity (36-39) and, in a recent report, to prevent TNF $\alpha$ -induced hemorrhagic necrosis of a transplanted Meth A sarcoma in BALB/c mice (40). The TNF-antagonizing effects of the soluble receptor fragments *in vitro* and *in vivo* imply a specific interaction with TNF $\alpha$  and TNF $\beta$  which might be an important regulatory mechanism of TNF action.

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<sup>1</sup> The abbreviations used are: TNF, tumor necrosis factor; TNFR $\alpha$ , 75-kDa TNF receptor; TNFR $\beta$ , 55-kDa TNF receptor; rsTNFR $\beta$ , recombinant soluble TNFR $\beta$ ; rsTNFR $\beta$ -h $\gamma$ 3, recombinant soluble TNFR $\beta$ -human IgG C $\gamma$ 3 fusion protein; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary.

In the present work a recombinant soluble form of the 55-kDa TNF receptor (rsTNFR $\beta$ ) was produced in high yields in different eukaryotic expression systems. The rsTNFR $\beta$  was also expressed as a human IgG C $\gamma$ 3 fusion protein (rsTNFR $\beta$ -h $\gamma$ 3) in myeloma cells. The recombinant receptor molecules were found to bind stoichiometrically to TNF $\alpha$  and TNF $\beta$  trimers and to neutralize TNF bioactivity in different assay systems.

## EXPERIMENTAL PROCEDURES

**Cell Lines and Reagents**—The *Spodoptera frugiperda* (Sf9) cell line was obtained from American Type Culture Collection (ATCC CRL 1711). The baculovirus *Autographa californica* (AcNP virus) was obtained from M. Summers, Texas A & M University, the Chinese hamster ovary (CHO)/dhfr<sup>-</sup> cell line from P. Familetti, Hoffmann-LaRoche Ltd., Nutley, NJ, and the WEHI164 (clone 2A3) cell line from J. R. Frey (51). The mouse myeloma cell line J558L was kindly provided by A. Trautnecker, Basel Institute of Immunology. The expression vector used to construct the rsTNFR $\beta$ -h $\gamma$ 3 fusion protein was modified from a CD4-immunoglobulin construct obtained from K. Karjalainen and A. Trautnecker (44). Recombinant human TNF $\alpha$  and TNF $\beta$  and mouse TNF $\alpha$  produced in *Escherichia coli* were kindly provided by W. Hunziker, H.J. Schoenfeld, and E. Hochuli (Hoffmann-LaRoche Ltd., Basel). Radioiodination of TNF $\alpha$  and TNF $\beta$  was performed with Na<sup>125</sup>I and Iodo-Gen (Pierce Chemical Co.) as described (25). For affinity column chromatography TNF $\alpha$  was coupled to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc.) according to the guidelines of the manufacturer. Protein G-Sepharose 4 Fast Flow was purchased from Pharmacia. Latex beads (polystyrene microspheres, 0.48  $\mu$ m diameter) were originally obtained from Polysciences, Inc., Warrington, PA and kindly provided by R. Spinnler and M. Caravatti (Hoffmann-LaRoche Ltd., Basel, Diagnostic Division).

**Construction of Vectors, Expression, and Purification**—The cDNA encoding the extracellular domain of TNFR $\beta$ , including the signal peptide, was amplified by the polymerase chain reaction. Unique restriction sites were introduced at both ends of the fragment. In addition, a translational stop codon was introduced behind the last amino acid of the extracellular domain (Thr<sup>182</sup>, numbering according to Ref. 27). The engineered fragment was cloned into an expression vector for mammalian cells. The plasmid contained the Rous sarcoma virus long terminal repeat and the 3' intron plus the polyadenylation site from the rat preproinsulin gene. The expression cassette was finally inserted into the *PvuII* restriction site of plasmid pSV2-DHFR. Transfected CHO/dhfr<sup>-</sup> cells were initially selected by the neomycin analogue G418 in  $\alpha$ -medium containing 200 nmol/ml methotrexate. Thereafter, the concentration of methotrexate was sequentially increased by 2–5-fold increments up to 150  $\mu$ mol/ml. For expression in the baculovirus system, homologous recombination was used to introduce the amplified cDNA fragment into the genome of the AcNP virus. Sf9 cells were grown at 27 °C in EX-CELL 400 medium (J. R. Scientific, Woodland, CA) containing 2% fetal bovine serum. Cell culture and viral infection were carried out as described (45). The recombinant viruses were purified by limited dilutions in microtiter plates followed by dot blot hybridization. The rsTNFR $\beta$ -h $\gamma$ 3 fusion protein was constructed by exchanging the CD4 sequence in the pCD4-h $\gamma$ 3-4 vector (44) with the TNFR $\beta$  extracellular domain sequence using *SacI* restriction sites. This procedure yielded a chimeric protein in which the TNFR $\beta$  sequence was inserted in front of the hinge region of the human IgG C $\gamma$ 3 chain. J558L mouse myeloma cells transfected with the rsTNFR $\beta$ -h $\gamma$ 3 construct by protoplast fusion were cultured in DHI medium (Dulbecco's modified Eagle's medium/Ham's F-12/Iscove's modified Dulbecco's medium, 25/25/50) supplemented with selenite (20 nM), ethanolamine (20  $\mu$ M), insulin (5  $\mu$ g/ml), human transferrin (6  $\mu$ g/ml), Primatone RL (2.5 mg/ml), Pluronic F68 (0.1 mg/ml), and 0–2% fetal calf serum (46). Expression of rsTNFR $\beta$  and rsTNFR $\beta$ -h $\gamma$ 3 was analyzed by a sandwich-type binding assay using radiolabeled or peroxidase-labeled TNF $\alpha$  and the non-neutralizing monoclonal antibody htr-20 (25).

Cell-free supernatants of cell transfectant cultures containing rsTNFR $\beta$  or rsTNFR $\beta$ -h $\gamma$ 3 were concentrated 5–10-fold by ultrafiltration (molecular mass cutoff of 10 kDa) and clarified by centrifugation and filtration through a 0.45- $\mu$ m filter. The clear filtrate was applied to a TNF $\alpha$  affinity column (Sf9 and CHO/dhfr<sup>-</sup> supernatants) or protein G affinity column (J558L supernatants). After extensive washing with phosphate-buffered saline (PBS) the columns

were eluted with 0.1 M glycine, 100 mM NaCl, pH 2.6, buffer. The fractions containing the recombinant proteins were concentrated and subjected to gel filtration chromatography on TSK3000SW or Superose 12 (Pharmacia) columns with PBS as solvent. The amount of protein was determined by amino acid analysis or BCA assay (Pierce). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described (47) using the mini-gel system of Bio-Rad.

**Binding Assay and Scatchard Analysis**—A 96-well microtiter plate coated with the TNFR $\beta$ -specific non-neutralizing monoclonal antibody htr-20 (25) was incubated with 10 ng/ml rsTNFR $\beta$  or rsTNFR $\beta$ -h $\gamma$ 3 in 1% defatted milk powder for 3 h at room temperature. Under these conditions only about 10% of the total binding sites were occupied by the receptor protein as determined from a titration curve (low density packing). In some experiments the antibody-coated plate was incubated with 3  $\mu$ g/ml soluble receptor to saturate all receptor binding sites (high or maximum density packing). After washing with PBS the wells were incubated with different concentrations of radiolabeled TNF $\alpha$  or TNF $\beta$  (1–250 ng/ml) in the presence or absence of a 200-fold excess unlabeled ligand for 16 h at 4 °C. The radioactivity bound to single wells was directly counted in a  $\gamma$ -counter. Nonspecific binding was subtracted.  $K_d$  values were determined from Scatchard plots.

**Quasielastic Light Scattering and Ultracentrifugation Analysis**—Quasielastic light scattering experiments were performed with the system ALV-300 (ALV Laser-Vertriebsgesellschaft, Langen, Germany). Samples of 300  $\mu$ l were filtered through 0.2- $\mu$ m filters in closed cylindrical quartz cells. The protein concentration was 0.5–1 mg/ml. Correlation functions were analyzed with the program CONTIN (48) that yields a distribution of relaxations. Mean values for the diffusion coefficient  $D$  were calculated assuming either an extended, i.e. rod-like (0 moment of the observed distribution) or roughly spherical structure (3rd moment) of the particles.

A Beckman Model E centrifuge with a AnD rotor and a 12-mm double sector Epon cell was used for analytical centrifugation studies. The rotor was run at 56,000 rpm in the sedimentation velocity experiments and at 24,000 or 11,000 rpm in the sedimentation equilibrium experiments. All runs were performed at room temperature using aliquots of the solutions investigated by quasielastic light scattering. Relative mole masses were calculated from the observed sedimentation velocities by the Svedberg equation using the mean values of the diffusion coefficients as described above. The partial specific volume of rsTNFR $\beta$  was assumed to be 0.68 ml/g taking into account 30% glycosylation (w/w) (49). Alternatively, the molecular masses were also obtained from the sedimentation equilibrium runs by analyzing the absorption as a function of the square radius (50).

**Competitive Inhibition of Ligand Binding to Native TNFR $\beta$  and TNFR $\alpha$  Holoreceptors**—1–2 ng of native TNFR $\beta$  and TNFR $\alpha$  purified from HL60 cells (26) were spotted to pretreated nitrocellulose membranes. After blocking with a solution of 1% defatted milk powder, the membrane was incubated with human radiolabeled TNF $\alpha$  or TNF $\beta$  (1 pmol/ml) in the presence of different concentrations of rsTNFR $\beta$  or rsTNFR $\beta$ -h $\gamma$ 3 for 2 h at room temperature. The membrane was then thoroughly rinsed with PBS and counted in a  $\gamma$ -counter.

**WEHI164 Cytotoxicity Assay**—WEHI164 cells (clone 2A3, kindly provided by J. R. Frey (51)) were cultured in a microtiter plate at 10<sup>4</sup> cells/well in a RPMI-based medium in the presence of human TNF $\alpha$  or TNF $\beta$  and different concentrations of rsTNFR $\beta$  or rsTNFR $\beta$ -h $\gamma$ 3 for 48 hours at 37 °C. Cell viability was determined by a dye uptake method as described earlier (9).

**Agglutination of Latex Beads**—5 mg of Latex beads washed with PBS, pH 5.0, buffer and H<sub>2</sub>O were incubated with 250  $\mu$ g of rsTNFR $\beta$ -h $\gamma$ 3 in 0.5 ml of PBS, pH 5.0, overnight at 4 °C on a rotating wheel. The beads were then treated with a solution of 1% defatted milk powder to block any remaining binding sites and washed with PBS buffer. To induce agglutination the beads were suspended at 0.2–1.0 mg/ml in PBS, pH 7.4, containing 0.1 mg/ml bovine serum albumin and 0.1% NaN<sub>3</sub>. Human TNF $\alpha$  was added at different concentrations and after overnight incubation at room temperature agglutination was analyzed in a light microscope at  $\times$  400 magnification.

## RESULTS

**Expression, Purification, and Ligand Binding Affinities of rsTNFR $\beta$** —Sf9 insect cells infected with the recombinant baculovirus secreted 5–10  $\mu$ g/ml of soluble receptor into the

medium after 3–5 days in culture. Transfected CHO/dhfr<sup>-</sup> cells produced up to 30 µg/ml of the recombinant protein after amplification in the presence of increasing methotrexate concentrations. The TNFRβ-hγ3 fusion protein was expressed and secreted in mouse myeloma cells with a yield of about 0.5–1 µg/ml.

The recombinant soluble TNF receptors were purified by TNFα or protein G affinity chromatography and gel filtration. SDS-PAGE analysis revealed for the baculovirus expressed protein three to four discrete bands between 21 and 25 kDa. When virus-infected Sf9 cells were cultured in the presence of tunicamycin, however, a single protein species of 21 kDa was obtained (see Fig. 1) which also was the only TNFα-reacting band in a ligand blot experiment (not shown). N-terminal sequence analysis of the glycosylated baculovirus-produced material revealed a single sequence starting with Leu<sup>1</sup> of the mature TNFRβ (not shown). rsTNFRβ produced in CHO/dhfr<sup>-</sup> cells yielded two bands migrating on SDS gels at around 28 and 32 kDa. Sequence analysis of this material confirmed the expected N terminus, but a second N-terminal sequence starting at Asp<sup>12</sup> was also present in a roughly 1:1 ratio. Interestingly, Asp<sup>12</sup> has previously been found to be the N terminus of the naturally occurring TNFRβ fragment (36). The TNFRβ-hγ3 fusion protein was expressed as a disulfide-linked homodimer indicating an antibody-like structure of this molecule. As shown in Fig. 1, reduced samples of baculovirus- or CHO/dhfr<sup>-</sup>-derived rsTNFRβ migrated at a slightly lower rate on SDS gels. This is most likely due to the high content of cysteines in these proteins. A similar observation has been made earlier with the native 55-kDa TNFRβ purified from HL60 cells (26).

The soluble receptor fragments produced in either expression system showed a high affinity for TNFα and a slightly lower affinity for TNFβ (see Fig. 2). The difference in the apparent  $K_d$  values of rsTNFRβ for TNFα and TNFβ was most prominent with the CHO/dhfr<sup>-</sup>-derived material. This finding is in contrast to the native cell surface-bound 55-kDa TNFRβ, which has been shown to bind both TNFα and TNFβ with about the same affinity, i.e.  $K_d$  values of 326 and 351 pM, respectively (24, 52). Interestingly, fully deglycosylated rsTNFRβ as expressed in baculovirus-infected Sf9 cells in the presence of tunicamycin displayed similar binding characteristics as the glycosylated form (data not shown), confirming that the carbohydrate moieties are not essential for ligand

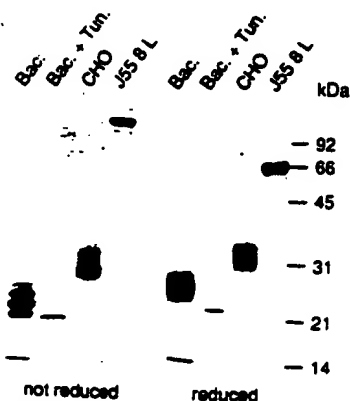


FIG. 1. SDS-PAGE analysis of purified rsTNFRβ and rsTNFRβ-hγ3. Purified rsTNFRβ and rsTNFRβ-hγ3 produced in different expression systems were separated by nonreducing and reducing SDS-PAGE and stained with Serva blue R. Expression systems: Bac., baculovirus-infected insect Sf9 cells; Bac. + Tun., baculovirus-infected insect cells grown in the presence of tunicamycin; CHO, CHO/dhfr<sup>-</sup> cells; J558L, mouse myeloma cells (expressing rsTNFRβ-hγ3).

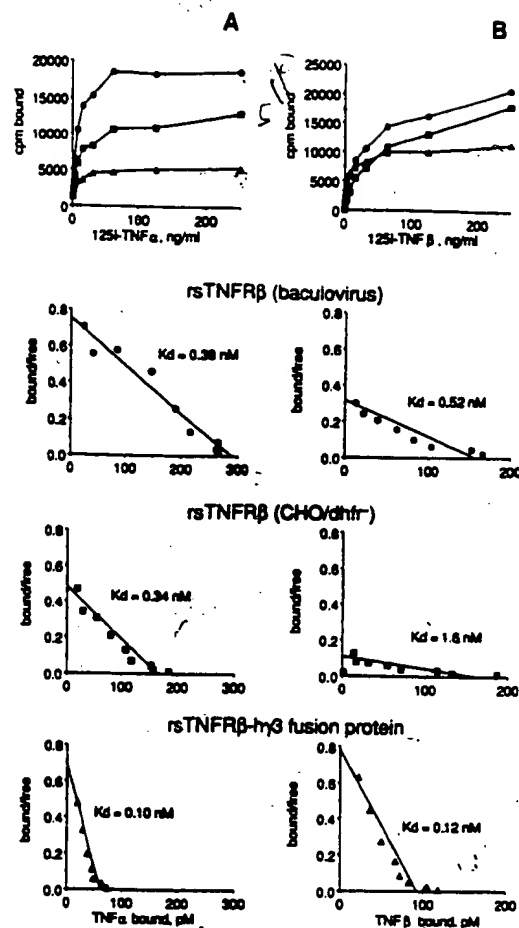


FIG. 2. Binding of TNFα and TNFβ to rsTNFRβ and rsTNFRβ-hγ3: binding curves and Scatchard analysis. Binding of <sup>125</sup>I-TNFα (A) and <sup>125</sup>I-TNFβ (B) to baculovirus-produced rsTNFRβ (circles), CHO/dhfr<sup>-</sup>-produced rsTNFRβ (squares), and rsTNFRβ-hγ3 fusion protein (triangles) was measured in a solid phase assay under low density packing conditions (see "Experimental Procedures"). The  $K_d$  values were determined from Scatchard analysis of the binding curves as indicated.

binding (24, 26). The apparent affinity of the bivalent rsTNFRβ-hγ3 fusion protein for TNFα and TNFβ was found to be significantly higher than the affinity of baculovirus- or CHO/dhfr<sup>-</sup>-derived monovalent rsTNFRβ (Fig. 2). It is interesting to note that  $K_d$  values determined in the solid phase assay under high receptor density conditions (see "Experimental Procedures") were generally higher and did not show a marked difference in the apparent affinities between the fusion protein and rsTNFRβ (data not shown). It therefore appears that at maximum dense packing of the solid phase some interactions of receptor molecules leading to multiple valency and/or steric constraints cannot be excluded.

**Stoichiometry of rsTNFRβ·TNFα and rsTNFRβ·TNFβ Complexes**—rsTNFRβ purified from CHO/dhfr<sup>-</sup> cell culture medium was incubated with TNFα or TNFβ at different receptor to ligand molar ratios and fractionated according to size by gel filtration chromatography. The chromatographic conditions chosen allowed to separate receptor-ligand complexes from free receptor and free ligand. As shown in Fig. 3, at an approximate 1:1 molar ratio neither free receptor nor free TNFα or TNFβ could be detected in the elution profiles indicating that under these conditions complete complex formation had occurred. Amino acid composition analysis of the separated complexes evaluated by a recently described computer program (53) confirmed the 1:1 stoichiometry (not

shown). When the amount of  $\text{TNF}\alpha$  added was gradually increased, a transition of the  $\text{TNFR}\beta \cdot \text{TNF}\alpha$  complex toward a slightly lower molecular mass was observed in the elution profile (Fig. 3, left panel). In contrast, adding increasing amounts of  $\text{TNF}\beta$  did not affect the elution behavior of the  $\text{TNFR}\beta \cdot \text{TNF}\beta$  complex (Fig. 3, right panel).

To obtain a more accurate molecular mass estimate of  $\text{rsTNFR}\beta$  and its complexes with  $\text{TNF}\alpha$  and  $\text{TNF}\beta$ , quasielastic light scattering and analytical ultracentrifugation studies were performed. The results are summarized in Table I. For  $\text{rsTNFR}\beta$  a monomeric structure with a molecular mass of 25 kDa was found by sedimentation equilibrium analysis. The theoretical molecular mass for  $\text{rsTNFR}\beta$  in its unglycosylated form is 20,467. Analysis of  $\text{rsTNFR}\beta$  complexed to  $\text{TNF}\alpha$  or  $\text{TNF}\beta$  under conditions of complete complex for-

mation (see a  $\text{TNF}\alpha$  added for both complexes a molecular mass of about 140 kDa. If a stoichiometry of three  $\text{rsTNFR}\beta$  molecules bound to one 49-kDa  $\text{TNF}\alpha$  or 57-kDa  $\text{TNF}\beta$  trimer (9) is assumed, theoretical molecular masses of 124 and 132 kDa, respectively, are calculated which are in approximate agreement with the observed values. Sedimentation velocity analysis combined with quasielastic light scattering data confirmed the molecular masses observed in the equilibrium runs and were, in addition, indicative for a rather extended, i.e. rod-like structure of  $\text{rsTNFR}\beta$  and its  $\text{TNF}\alpha$  and  $\text{TNF}\beta$  complexes.

**Inhibition of  $\text{TNF}\alpha$  and  $\text{TNF}\beta$  Binding by  $\text{rsTNFR}\beta$  and  $\text{rsTNFR}\beta\text{-h}\gamma 3$** — $\text{rsTNFR}\beta$  and  $\text{rsTNFR}\beta\text{-h}\gamma 3$  were tested for their ability to competitively inhibit binding of  $\text{TNF}\alpha$  and  $\text{TNF}\beta$  to native  $\text{TNFR}\alpha$  and  $\text{TNFR}\beta$  purified from HL60 cells. In this assay native highly purified receptors were spotted onto nitrocellulose membranes and incubated with  $^{125}\text{I}$ - $\text{TNF}\alpha$  or  $^{125}\text{I}$ - $\text{TNF}\beta$  in the presence of different concentrations of  $\text{rsTNFR}\beta$  or  $\text{rsTNFR}\beta\text{-h}\gamma 3$ . As shown in Fig. 4, A and C, binding of  $^{125}\text{I}$ - $\text{TNF}\alpha$  to both TNF receptors was blocked by  $\text{rsTNFR}\beta$  and  $\text{rsTNFR}\beta\text{-h}\gamma 3$  in a concentration-dependent manner. It is interesting to note that a roughly equimolar concentration of the fusion protein was sufficient to prevent  $\text{TNF}\alpha$  binding almost completely.  $\text{rsTNFR}\beta$  was about  $10\text{--}100 \times$  less potent in inhibiting the binding. The binding of  $^{125}\text{I}$ - $\text{TNF}\beta$  was also inhibited (Fig. 4, B and D), but higher concentrations of  $\text{rsTNFR}\beta$  and  $\text{rsTNFR}\beta\text{-h}\gamma 3$  were needed to achieve inhibitory effects comparable to  $\text{TNF}\alpha$ . The 10–15% residual binding seen with iodinated  $\text{TNF}\beta$  at high soluble receptor concentrations is due to nonspecific binding of radioactivity to the nitrocellulose filter.

The inhibition of TNF cytotoxicity by  $\text{rsTNFR}\beta$  and  $\text{rsTNFR}\beta\text{-h}\gamma 3$  was tested in a cellular cytotoxicity assay using the 2A3 subclone of the murine fibrosarcoma cell line WEHI164 (51). As expected from the binding studies,  $\text{rsTNFR}\beta\text{-h}\gamma 3$  very efficiently inhibited TNF activity; at a concentration of 0.1 pmol/ml, i.e. equimolar to the  $\text{TNF}\alpha$  concentration used in the assay,  $\text{rsTNFR}\beta\text{-h}\gamma 3$  prevented  $\text{TNF}\alpha$ -induced cytotoxicity very efficiently (Fig. 5A).  $\text{rsTNFR}\beta$  also had inhibitory activity but a concentration about 100-fold in excess of  $\text{TNF}\alpha$  was needed for complete inhibition.  $\text{TNF}\beta$ -induced cytotoxicity was also inhibited by the fusion protein, albeit not at equimolar concentrations. The protective effects of  $\text{rsTNFR}\beta$  in these cytotoxicity assays were only evident at rather high concentrations (Fig. 5B).

**$\text{TNF}\alpha$ -induced Agglutination of  $\text{rsTNFR}\beta\text{-h}\gamma 3$ -coated Latex Beads**—In view of the trimeric structure of  $\text{TNF}\alpha$  and  $\text{TNF}\beta$ , each capable of binding three recombinant soluble receptor molecules, it is very likely that these cytokines aggregate TNF receptors on the cell surface into microclusters which may be a necessary step in signal transduction. To mimic cell sur-

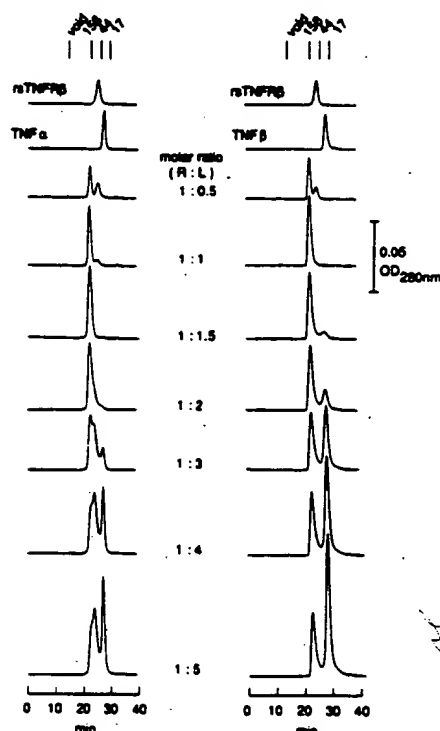


FIG. 3. Analysis of  $\text{rsTNFR}\beta$  and its  $\text{TNF}\alpha$  and  $\text{TNF}\beta$  complexes by gel filtration chromatography. 1 nmol of  $\text{rsTNFR}\beta$  purified from CHO/dhfr<sup>-</sup> cells was mixed with 0.5, 1, 1.5, 2, 3, 4, and 5 nmol of  $\text{TNF}\alpha$  or  $\text{TNF}\beta$  in 0.1 ml of PBS. (The amount of  $\text{TNF}\alpha$  and  $\text{TNF}\beta$  was calculated for the 17-kDa monomeric unit.) The mixtures with the various receptor:ligand (R:L) molar ratios were fractionated on a Superose 12 column (Pharmacia) in PBS. Numbers on top indicate the positions of molecular weight marker proteins (Bio-Rad). Left panel,  $\text{TNF}\alpha$  complexes; right panel,  $\text{TNF}\beta$  complexes.

TABLE I

Molecular mass determination of  $\text{rsTNFR}\beta$  and its complexes with  $\text{TNF}\alpha$  and  $\text{TNF}\beta$

Molecular masses were determined in the analytical ultracentrifuge as described under "Experimental Procedures." In the sedimentation velocity analyses the molecular masses were calculated according to the Svedberg equation using diffusion coefficients  $D$  determined in quasielastic light scattering measurements.

	Molecular mass (kDa)		Putative stoichiometry
	Sedimentation equilibrium	Sedimentation velocity <sup>a</sup>	
$\text{rsTNFR}\beta$	25	20: 32	Monomer
$\text{rsTNFR}\beta \cdot \text{TNF}\alpha$ complex	140	115: 156	$[\text{TNFR}\beta]_3 \cdot [\text{TNF}\alpha]$
$\text{rsTNFR}\beta \cdot \text{TNF}\beta$ complex	140	102: 139	$[\text{TNFR}\beta]_3 \cdot [\text{TNF}\beta]$

<sup>a</sup> The 3rd and 0 moments of  $D$  were used in the calculation yielding molecular masses for a roughly spherical (listed first) and an extended (listed second) structure, respectively.

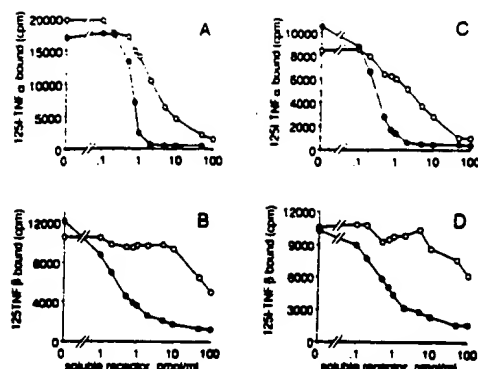


FIG. 4. Binding of  $^{125}\text{I}$ -TNF $\alpha$  and  $^{125}\text{I}$ -TNF $\beta$  to native TNFR $\beta$  and TNFR $\alpha$ : inhibition by rsTNFR $\beta$  and rsTNFR $\beta$ -h $\gamma$ 3. Binding inhibition to native full-length TNFR $\beta$  and TNFR $\alpha$  purified from HL60 cells was measured in a dot blot assay as described under "Experimental Procedures." The concentration of radiolabeled ligand in the assay was 1 pmol/ml. Open circles, ligand binding in the presence of increasing concentrations of rsTNFR $\beta$ ; closed circles, ligand binding in the presence of increasing concentrations of rsTNFR $\beta$ -h $\gamma$ 3. (The concentration of the rsTNFR $\beta$ -h $\gamma$ 3 homodimer was calculated for the 66-kDa monomer unit). A,  $^{125}\text{I}$ -TNF $\alpha$  binding to TNFR $\beta$ ; B,  $^{125}\text{I}$ -TNF $\beta$  binding to TNFR $\beta$ ; C,  $^{125}\text{I}$ -TNF $\alpha$  binding to TNFR $\alpha$ ; D,  $^{125}\text{I}$ -TNF $\beta$  binding to TNFR $\alpha$ .

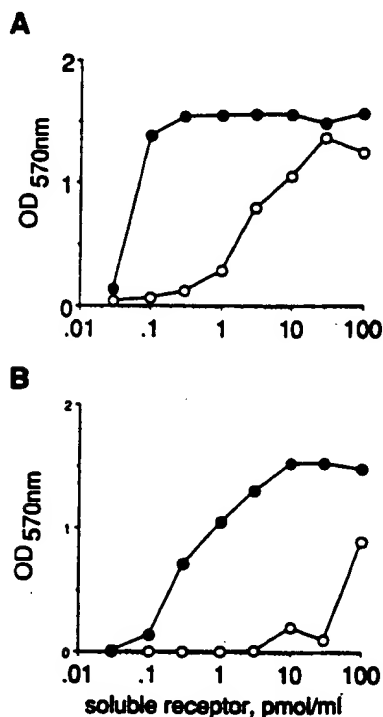


FIG. 5. Inhibition of TNF $\alpha$ - and TNF $\beta$ -induced cytotoxicity in WEHI164 cells. WEHI164 cells were cultured in the presence of 0.1 pmol/ml TNF $\alpha$  (A) or TNF $\beta$  (B) and different concentrations of rsTNFR $\beta$  (open circles) and rsTNFR $\beta$ -h $\gamma$ 3 (closed circles). Cell viability was analyzed after 48 h at 37 °C.

face-bound TNF receptors, Latex beads were coated with rsTNFR $\beta$ -h $\gamma$ 3 fusion protein and subsequently exposed to different concentrations of TNF $\alpha$ . TNF $\alpha$  induced an agglutination of rsTNFR $\beta$ -h $\gamma$ 3 as visualized by agglutination of the Latex beads (Fig. 6). A similar effect was seen with TNF $\beta$ , but agglutination was much less pronounced (results not shown).

#### DISCUSSION

In this study TNF binding and inhibiting properties of the extracellular region of the human TNFR $\beta$  were analyzed. The

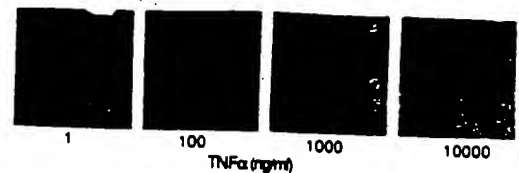


FIG. 6. Agglutination of rsTNFR $\beta$ -h $\gamma$ 3-coated Latex beads. Latex beads (0.48  $\mu\text{m}$  diameter) were coated with rsTNFR $\beta$ -h $\gamma$ 3 and incubated at 1 mg/ml with different concentrations TNF $\alpha$  as indicated. Agglutination of the beads was visualized in a light microscope at  $\times 400$  magnification.

recombinant soluble receptors (rsTNFR $\beta$  and rsTNFR $\beta$ -h $\gamma$ 3 fusion protein) expressed in different eukaryotic expression systems displayed high affinity binding to human TNF $\alpha$  similar to that of native cell surface-bound 55-kDa TNFR $\beta$ . In contrast, the binding affinity of rsTNFR $\beta$  for TNF $\beta$  was significantly decreased when compared with the native cell surface receptor. A similar observation, i.e. impaired neutralization of TNF $\beta$  versus TNF $\alpha$ , has also been made with a so-called TNF binding protein, which is a naturally occurring soluble receptor derived from TNFR $\beta$  (33, 36, 39). It therefore appears that with respect to ligand binding properties, rsTNFR $\beta$  closely resembles the natural TNF inhibitor. The apparent lower affinity of rsTNFR $\beta$  (and also of the detergent-solubilized holoreceptor (9)) for TNF $\beta$  might reflect a microenvironment of the ligand binding site which is slightly different from that of the cell surface-bound full-length TNF receptor. It is noteworthy that with respect to monovalent rsTNFR $\beta$  the rsTNFR $\beta$ -h $\gamma$ 3 fusion protein binds both TNF $\alpha$  and TNF $\beta$  with a severalfold higher affinity when measured under appropriate assay conditions. This increase in affinity most probably reflects a higher avidity of the rsTNFR $\beta$ -h $\gamma$ 3 construct due to its bivalency. Comparison of rsTNFR $\beta$  and the fusion protein to compete with native full-length TNF receptors for TNF binding and to protect WEHI 164 cells from TNF-induced cytotoxicity indeed confirmed the expected higher activity of the fusion protein.

The results from the ultracentrifugation analyses indicate that rsTNFR $\beta$  is monomeric in solution. The complexes of rsTNFR $\beta$  with TNF $\alpha$  or TNF $\beta$  both had a molecular mass of about 140 kDa which favors a stoichiometry of three rsTNFR $\beta$  monomers bound to one TNF $\alpha$  or TNF $\beta$  trimer. It has been proposed that the receptor binding site on the TNF $\alpha$  trimer is located at the boundary of two monomeric units near the base of the bell-shaped structure thus implying three potential receptor binding sites (7, 54). Such a model is fully compatible with the size of receptor-ligand complexes as determined in the present study. It is interesting to note that an intermediate lower molecular weight form of the rsTNFR $\beta$ -TNF $\alpha$  complex can be partially resolved by gel filtration when a slight excess of TNF $\alpha$  over rsTNFR $\beta$  is present. Most likely, this intermediate form represents TNF $\alpha$  trimers complexed to only one or two rsTNFR $\beta$  molecules. Such intermediate forms are not seen with rsTNFR $\beta$ -TNF $\beta$  complexes. Whether these distinct binding characteristics of TNF $\alpha$  and TNF $\beta$  are also true for cell surface-bound receptors remains to be elucidated.

The results of sedimentation velocity and quasielastic light scattering measurements indicate that rsTNFR $\beta$  and its TNF $\alpha$  and TNF $\beta$  complexes have a rather extended, i.e. rod-like structure. This conclusion is supported by the relative large apparent molecular masses of 62, 170, and 150 kDa for rsTNFR $\beta$ , rsTNFR $\beta$ -TNF $\alpha$  and rsTNFR $\beta$ -TNF $\beta$  complexes, respectively, determined by gel filtration chromatography. A similar relatively large apparent molecular mass (50 kDa) has been found for the natural soluble TNFR $\beta$  on sizing columns (42).

Soluble fragments of bo  $\alpha$  and TNFR $\alpha$  are found *in vivo*. They are present at relatively high concentrations in normal human serum and urine but can be drastically increased in certain disease states.<sup>2</sup> The cellular source and the mechanism of receptor shedding remain unclear. It has been speculated that soluble TNF receptor fragments might participate in the control of TNF $\alpha$  and/or TNF $\beta$  toxicity by neutralization and rapid clearance of systemic TNF $\alpha$  and TNF $\beta$  (33, 36, 37, 39, 42). However, the fact that at least a 10-fold excess of the soluble receptor with respect to TNF $\alpha$  (and more than a 100-fold excess with respect to TNF $\beta$ ) is needed to obtain a significant neutralization demonstrates that the neutralizing capacity of serum is restricted. The rsTNFR $\beta$ -h $\gamma$ 3 construct as described in this study, therefore, is a promising TNF antagonizing agent for neutralization of systemic TNF toxicity in certain disease states.

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<sup>2</sup> M. Brockhaus and H. Gallati, unpublished data.